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B7H1/CD80 Interaction Augments PD-1–Dependent T Cell Apoptosis and Ameliorates Graft-versus-Host Disease

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Interactions of B7H1 (programmed death ligand 1 [PD-L1]) with its two ligands, PD-1 and CD80, on T cells play a pivotal role in controlling T cell activation, proliferation, anergy, and apoptosis. However, the interactions between the two pathways remain unknown. Using an autoimmune response model of graft-versus-host disease (GVHD), we report in this study that: 1) Comparison of proliferation and apoptosis of wild-type (WT) and PD-1−/−/CD4+ conventional T (Tcon) cells in WT and B7H1−/− recipients revealed that B7H1/CD80 interaction per se augments T cell proliferation, and this interaction augments T cell apoptosis mediated by B7H1/PD-1 interaction. This observation was recapitulated in an in vitro MLR assay. 2) Specific blockade of the B7H1/CD80 axis by anti-B7H1 mAb reduces WT-alloreactive Tcon cell proliferation, IL-2 production, expression of PD-1, and apoptosis, resulting in worsening GVHD. In contrast, specific blockade of B7H1/CD80 interaction reduces donor PD-1−/− Tcon cell proliferation without an impact on apoptosis, resulting in ameliorating GVHD. 3) B7H1 fused to an Ig Fc domain (B7H1-Ig), when produced in vivo by hydrodynamic injection of B7H1-Ig plasmid, ameliorates GVHD by augmenting proliferation and apoptosis of WT alloreactive Tcon cells. Conversely, B7H1-Ig treatment has no impact on apoptosis but augments PD-1−/− T cell proliferation and worsens GVHD. These results indicate that B7H1/CD80 interaction augments Tcon cell proliferation, IL-2 production, and expression of PD-1, which leads to increased apoptosis mediated by the B7H1/PD-1 pathway. Additionally, by engaging both PD-1 and CD80, B7H1-Ig can be a powerful therapeutic reagent for downregulating the T cell immune response. The Journal of Immunology, 2015, 194: 000–000.

The activation status of T cells and the level of immune responses are controlled by costimulatory and coinhibitory molecules, although the Ag specificity is decided by interactions between TCR and MHC–peptide complex (1). Many costimulatory (i.e., CD28, ICOS, and OX40) and coinhibitory (i.e., CTLA-4, programmed death 1 [PD-1], and CD80) molecules have been described on T cells (1, 2). B7H1 (also known as PD ligand 1 [PD-L1]) is a ligand for both PD-1 and CD80. B7H1 is constitutively expressed by APCs, such as dendritic cells (DCs), and its expression is further increased upon cell activation; B7H1 expression on nonhematopoietic cells, such as parenchymal cells, is induced by inflammatory cytokines (i.e., IFN-γ) (3–8). PD-1 expression on T cells is strongly induced upon T cell activation (2, 8–12). CD80 is expressed by naïve T cells and upregulated upon activation (13). B7H1 interaction with its ligands expressed by hematopoietic cells such as DCs and nonhematopoietic cells have been previously reported to play crucial roles in T cell activation, apoptosis, anergy, and exhaustion (6, 8, 14–18). B7H1/PD-1 interaction has been recently reported to inhibit T cell cycle progression and effector function (19). However, the impact of B7H1/CD80 interaction per se on T cells remains unclear; how B7H1/PD-1 and B7H1/CD80 pathways interact also remains unknown.

B7H1 interactions with both PD-1 and CD80 are required for induction of peripheral T cell tolerance, and blockade of either one can prevent induction of tolerance or augment autoimmune (16, 20–22). Blockade of B7H1/PD-1 interaction via anti–PD-1 or anti-B7H1 mAb has recently been shown to augment antitumor immunity in mice (23) and patients with B7H1-expressing cancers (24–26). This clinical success has demonstrated the importance of B7H1/PD-1 interaction in downregulating cancer immunity. Alternatively, there have been contradicting reports regarding how B7H1 (PD-L1) regulates T cell immune responses in autoimmune. Tissue expression of B7H1 (PD-L1) was shown to protect against insulitis in type 1 diabetes in NOD mice (27, 28) and suppress graft-versus-host disease (GVHD) (6, 8). In contrast, expression of B7H1 transgene by islet β cells was reported to induce autoimmune insulitis and augment islet graft rejection (29). There have also been conflicting reports on the effect of treatment with B7H1-Ig, which is a protein consisting of the extracellular domain of B7H1 fused with an Ig Fc domain. Whereas B7H1-Ig was reported to augment human T cell proliferation and production.
of IL-10 (3, 30). B7H1-Ig has also been reported to reduce human and murine T cell proliferation and reduce IL-10 production in different settings (4, 31). B7H1-Ig was shown to ameliorate cardiac allograft rejection (32), whereas others have found that B7H1-Ig augments islet graft rejection (29). Thus, it remains unclear whether B7H1-Ig can effectively downregulate auto- and alloimmunity.

In the present study, using an alloimmune response model of acute GVHD, we investigated the role of B7H1/CD80 interaction on alloreactive conventional CD4+ T (Tcon) cell proliferation and apoptosis. We also investigated the interactions between B7H1/CD80 and B7H1/PD-1 pathways. We have observed that B7H1/CD80 interaction per se augments Tcon cell proliferation without an impact on apoptosis and augments GVHD; we have also observed that B7H1/CD80 interaction augments Tcon cell apoptosis in the presence of B7H1/PD-1 interaction and ameliorates GVHD. Additionally, in vivo–produced agonistic B7H1-Ig augments Tcon cell apoptosis in the presence of PD-1 and ameliorates GVHD. Conversely, in the absence of PD-1, B7H1-Ig augments Tcon cell proliferation without impact on Tcon cell apoptosis and augments GVHD. These studies not only reveal a novel role of B7H1/CD80 in regulating T cell proliferation and apoptosis in a B7H1/PD-1–dependent manner but also provide explanations to previous contradictory reports about the role of B7H1-Ig in regulating autoimmunity.

Materials and Methods

Mice
Wild-type (WT) BALB/c (H-2b) mice were purchased from National Cancer Institute Laboratories (Frederick, MD). B7H1−/− BALB/c mice were established as previously described (14). PD-1−/− C57BL/6 breeders were obtained from Dr. Tasuku Honjo’s laboratory (Kyoto University, Kyoto, Japan). GFP-Foxp3 C57BL/6 (H-2b) was obtained from Dr. K. Kuchroo (Brigham and Women’s Hospital, Harvard Medical School, Boston, MA). Mice were maintained in a pathogen-free room at the City of Hope Research Animal Facilities (Duarte, CA). All animal protocols were approved by the City of Hope Research Animal Care Committee.

Induction and assessment of GVHD
Bone marrow and spleen harvest as well as hematopoietic cell transplantation (HTC) procedures were described in our previous publication (8). In brief, recipient mice (6–12 wk of age) were given 800 cGy total body irradiation (TBI) from a 137Cs source and then injected with CD25−CD8− spleen cells (2.5 × 10⁶) and T cell–depleted bone marrow (TCD-BM) cells (2.5 × 10⁶) from GFP-Foxp3 C57BL/6 (H-2b) donor mice or CD25−CD8− spleen cells (0.25 × 10⁶) and TCD-BM cells (2.5 × 10⁶) from PD-1−/− C57BL/6 donors. Thereafter, recipient mice were monitored for clinical GVHD and survival as described in our previous publications (8). The experimental and control groups were set up at the same time and experiments were replicated three times.

Plasmid and hydrodynamic injection
Control Fc and B7H1-Fc–expressing plasmids were produced by Dr. Lieping Chen’s laboratory (Yale University School of Medicine). B7H1−/− BALB/c mice were injected with plasmid in PBS through the tail vein of a CMV promoter. Both the control-Fc (human IgG1 CH2-CH3 domain) and B7H1-Fc vectors were delivered into B7H1−/− BALB/c mice by hydrodynamic injection as previously described (8, 34, 35). Briefly, B7H1−/− BALB/c mice were injected with plasmid in PBS through the tail vein of a CMV promoter. Both the control-Fc (human IgG1 CH2-CH3 domain) and B7H1-Fc vectors were delivered into B7H1−/− BALB/c mice by hydrodynamic injection as previously described (8, 34, 35). Briefly, when donor T cells were fully activated ~5 d after HCT, as judged by increased CD44 expression and decreased CD62L expression, recipients were injected daily with anti-B7H1 (43H12) or control rat IgG at a dose of 200 μg/mouse. Recipients receiving transplants from WT C57BL/6 donors were given two daily injections. Recipients receiving transplants from PD-1−/− C57BL/6 donors were given six to seven daily injections.

mAbs, flow cytometric analysis, and cell sorting
mAbs to mouse CD4, CD8, and H-2k, were all purchased from BD Pharmingen (San Diego, CA). Multiple-color FACS analysis was performed at the City of Hope FACS facility using a three-laser Cyan immunocytometry system (DakoCytomation, Fort Collins, CO), and data were analyzed using FlowJo software (Tree Star, San Carlos, CA) as previously described (36). Donor CD4+ T cells were stained with donor marker H-2k anti-mouse CD4+ and sorted using a BD FACSaria III sorter (BD Biosciences, San Jose, CA) at the City of Hope FACS facility. The sorted cells were used for total RNA and protein isolation.

In vivo BrdU labeling and annexin V staining
Day 3 T cell proliferation after HCT was measured by daily i.p. injection of BrdU (2.5 mg/mouse, ∼100 μg/g) 72 h before tissue harvesting. Day 6 T cell proliferation after HCT was measured with one i.p. injection with BrdU (2.5 mg/mouse, ∼100 μg/g) 3 h before tissue harvesting. Analysis of donor CD4+ T cells for BrdU incorporation was performed according to the manufacturer’s instructions (BD Pharmingen). For annexin V staining, the percentage of annexin V+ cells among donor CD4+ T cells was assessed by flow cytometry according to the manufacturer’s instructions (eBioscience, San Diego, CA).

Real-time RT-PCR
The process of real-time RT-PCR analysis of mRNA for PD-1, Bcl-xL, caspase-3, IL-2, IL-10, IFN-γ, and TNF-α in B7H1 were performed as described in our previous publication (13, 37). Primers used are as follows: Bcl-xL forward, 5′-GCTGCGGAGACTTCTGTGAAG-3′, reverse, 5′-TGCTGTGACTCITCCTCCAGT-3′; caspase-3, forward, 5′-ATGGAGCAGATTGAGTGGTGG-3′, reverse, 5′-CTCATGTGTTTCTAGGAGG-3′; IL-2, forward, 5′-TGAGCAATGGAGAATGGGGG-3′, reverse, 5′-GCTCCAATGTGGAATTCTGGC-3′; IL-10, forward, 5′-ACTGCGAACCCTCTCCAGT-3′, reverse, 5′-TGTCACGTTCCGTTTTTGTGG-3′; IFN-γ, forward, 5′-ACTGGCAGAAGAGGATGGTGG-3′, reverse, 5′-GAGCTTGTGTTGTGAGCCT-3′; TNF-α, forward, 5′-AGGCCCTGCTTGTAGC-3′, reverse, 5′-CTCCCTTTTGCCAGAACATCTGGG-3′, PD-1, forward, 5′-ACCCTGTTGATCCTACCCG-3′, reverse, 5′-ATTGTC CCTCCTGACCAAC-3′; B7H1, forward, 5′-GCTTCTACAATCCTTCA-3′, reverse, 5′-ACAAATGGAAATCGCTCTTGT-3′; CD80, forward, 5′-ACAACCTGGAACATGACCTTCTTTCC-3′, reverse, 5′-CATGGTGCTGTTTGGACCT-3′.

Western blot
Sorted donor CD4+ T cells (2 × 10⁶) were lysed in RIPA buffer supplemented with phosphatase inhibitors and protease inhibitors obtained from Santa Cruz Biotechnology (Dallas, TX). Cell lysate (20 μg) was fractionated by SDS-PAGE and blotted onto polyvinylidene difluoride. Membranes were blocked in 5% nonfat dry milk in TBST incubated with specific primary Abs, rabbit anti-mouse caspase-3 that can bind both total and cleaved caspase-3, rabbit anti-mouse Bcl-xL, or rabbit anti-mouse β-actin at 4°C overnight. All of these Abs were purchased from Cell Signaling Technology (Danvers, MA). The membrane was washed with TBST and incubated with goat anti-rabbit IgG and developed with the ECL chemiluminescent substrate (Thermo Fisher Scientific, Rockford, IL).

Intracellular cytokine staining
Intracellular cytokine staining for IL-2, TNF-α, IFN-γ, and IL-10 was performed as described in our previous publications (7, 38). Anti-mouse IL-2, IL-10, IFN-γ, and TNF-α were all purchased from BD Pharmingen.

Measurement of cytokines in serum
Serum concentrations of cytokines were determined by ELISA kits following the manufacturer’s instructions. The ELISA kits for IL-2 and TNF-α were obtained from eBioscience, whereas kits for IFN-γ and IL-10 were from Thermo Fisher Scientific.

MLR assay
Sorted CD25−CD4+ Tcon cells from WT or PD-1−/− C57BL/6 mice were labeled with CFSE (2.5 μM), and after washing labeled Tcon cells (0.1 × 10⁶) were cocultured with irradiated CD11c+ DCs (0.1 × 10⁶) from WT or B7H1−/− BALB/c mice. Three days after stimulation, cells were harvested.
for flow cytometry analysis of proliferation and apoptosis. Supernatants were measured for IL-2 concentration using ELISA.

**Statistical analysis**

Body weight and survival between groups were compared using the log-rank test (GraphPad Prism version 6.0). Unpaired two-tailed Student’s t tests were used to compare means. A p value < 0.05 was considered statistically significant.

**Results**

**Lack of host tissue expression of B7H1 reduces both proliferation and apoptosis of alloreactive donor CD4+ Tcon cells, but reduction of apoptosis outweighs reduction of proliferation, resulting in expansion of Tcon cells and worsening GVHD**

We recently reported that the interaction of B7H1 with CD80 (B7H1/CD80) augmented Foxp3'CD4' regulatory T (Treg) cell survival in allogeneic HCT recipients (13). However, the effect of B7H1/CD80 interaction on alloreactive CD4' Tcon cell proliferation and apoptosis remains unclear, and the impact of B7H1/CD80 interaction on B7H1/CD80 interaction is also unclear. Using a GVHD model of C57BL/6 donor to MHC-mismatched BALB/c recipient described in our previous publications (37, 39, 40), we first compared the proliferation and apoptosis of donor CD4' Tcon cells and GVHD severity in WT and B7H1' recipients. Because B7H1 can be expressed by host hematopoietic cells and parenchymal cells after HCT, as described in our previous publications (8, 13), and because donor Tcon cells can interact with B7H1 expressed by either or both cells, in the present study we evaluated the general effect of donor Tcon cell interaction with B7H1 expressed by both hematopoietic and parenchymal cells, and we use the term B7H1/CD80 interaction to indicate the absence of host tissue expression of B7H1.

To avoid the impact of CD25'CD4'Foxp3' Treg and CD8' T cells, we depleted splenocytes (CD25'CD8' SPLL) that contained ~15% CD4' Tcon cells from Foxp3-GFP C57BL/6 donors were used for transplantation. The residual Foxp3-GFP' cells among CD4' T cells was <1%, and CD8' T cells among CD25'CD8' SPLL was <0.2% (Supplemental Fig. 1A). CD25'CD8' SPLL (2.5 x 10^6) and TCD-BM cells (2.5 x 10^6) from donors were transplanted into sublethally irradiated WT or B7H1' recipients. WT BALB/c recipients showed slow body weight loss, and ~50% of them survived for >50 d after HCT; in contrast, B7H1' recipients showed rapid body weight loss and all died within 7 d (p < 0.01, Fig. 1A, 1B). The more severe GVHD in B7H1' recipients was not due to differences in Treg cell expansion, because there were fewer Foxp3' Treg cells among donor CD4' T cells in the spleen before HCT or 6 d after HCT in both recipients (Supplemental Fig. 1). This is also consistent with our previous publication that showed that few Treg cells were generated from conversion of Tcon cells early after HCT (13).

We also compared donor CD4' Tcon cell yield, proliferation, and apoptosis in the spleen and liver, a GVHD target organ, by measuring in vivo BrdU labeling and mexin V staining. Because T cell proliferation during the first 3 d after HCT was weak and it became very strong by 6 d after HCT, as previously reported (41, 42), we labeled T cells with BrdU for 72 h for the first 3 d and only for 3 h on day 6. We found that CD4' Tcon cell yield in the spleen of B7H1' recipients was significantly lower 3 d after HCT as compared with WT recipients (p < 0.05, Fig. 1C). The reduced Tcon cell yield in the spleen of B7H1' recipients was associated with significantly reduced proliferation of Tcon cells (p < 0.05, Fig. 1D, upper panel), although apoptosis of Tcons was similar (Fig. 1D, lower panel).

However, by 6 d after HCT, the CD4' Tcon cell yield was significantly increased in the spleen and liver of B7H1' recipients, as compared with WT recipients (p < 0.05, Fig. 1E, 1G). The increased Tcon cell yield in B7H1' recipients was associated with significant reduction of Tcon cell apoptosis, as judged by decreased percentage of annexin V' Tcon cells in both spleen and liver of B7H1' recipients as compared with WT recipients (p < 0.001, Fig. 1F, 1H). The Tcon cell proliferation in the B7H1' recipients was still lower, as judged by significant decrease of BrdU' Tcon cells in the spleen and liver of B7H1' recipients, as compared with WT recipients (p < 0.01, Fig. 1F, 1H). These results indicate that lack of host tissue expression of B7H1 (including hematopoietic cells and nonhematopoietic cells) leads to reduction in proliferation and apoptosis of alloreactive CD4' Tcon cells. The reduction in apoptosis of activated T cells appears to outweigh the reduction in T cell proliferation, as the lack of host tissue expression of B7H1 ultimately results in an accumulation of donor Tcon cells in both spleen and liver and exacerbation of GVHD. It is of interest that reduction of donor Tcon cell proliferation is associated with reduction of apoptosis in the absence of host tissue expression of B7H1.

**Lack of host tissue expression of B7H1 reduces proliferation with no influence on apoptosis of PD-1' alloreactive donor CD4' Tcon cells, resulting in reduction of expansion of Tcon cells and ameliorating GVHD**

Because the interaction of B7H1 with PD-1 mainly suppresses T cell cycle progression of activated T cells (19), the above observation of reduction of T cell proliferation in B7H1' hosts most likely resulted from the disruption of B7H1/CD80 interaction. Thus, we further tested the role of B7H1/CD80 interaction on the proliferation and apoptosis of Tcon cells by transplanting PD-1' Tcon cells into WT and B7H1' recipients. First, we found that donor PD-1' CD4' Tcon cells were much more potent than were WT CD4' Tcon cells in inducing acute GVHD. Whereas recipients that received CD25'CD8'SPLL (2.5 x 10^6) from PD-1' C57BL/6 donors all died within 7 d, ~60% of recipients receiving WT C57BL/6 donor cells survived for >50 d (p < 0.01, Supplementary Fig. 2). When the disease severity is too strong, it is difficult to determine the effect of amelioration or exacerbation of GVHD. Therefore, small numbers (0.25 x 10^6, 0.1 of the regular dose) of PD-1' CD25'CD8' donor spleen cells were transplanted. We found that PD-1' donor cells induced more rapid weight loss and death in WT recipients as compared with B7H1' recipients (p < 0.01, Fig. 2A, 2B). The more severe GVHD in WT recipients resulted from expansion of Tcon cells in both spleen and liver tissues (p < 0.01, Fig. 2C, 2E), which was associated with increased proliferation (p < 0.01) with no difference in apoptosis (Fig. 2D, 2F). These results indicate that in the absence of PD-1 on T cells, B7H1/CD80 interaction augments T cell proliferation without an impact on T cell apoptosis.

**Blockade of B7H1/CD80 interaction in the presence of PD-1 reduces proliferation and apoptosis of alloreactive donor CD4' Tcon cells, but reduction of apoptosis outweighs reduction of proliferation, resulting in expansion of Tcon cells and worsening GVHD**

Next, we attempted to further test the role of B7H1/CD80 interaction on donor T cell proliferation and apoptosis. We cannot use CD80' donor CD4' T cells to definitively evaluate the role of B7H1/CD80 interaction in Tcon cell proliferation and apoptosis, as CD80 on T cells also interacts with CD28 and CTLA-4 (43). Thus, we took advantage of a special anti-B7H1 mAb (43H12) that specifically blocks B7H1/CD80 interaction while leaving
FIGURE 1. Lack of host tissue expression of B7H1 results in reduced proliferation and apoptosis of alloreactive donor CD4+ Tcon cells and worsens GVHD. TBI-conditioned BALB/c or B7H1−/− BALB/c recipients were transplanted with CD25−CD8− splenic (2.5 × 10^6) and TCD-BM cells (2.5 × 10^6) from GFP-Foxp3 C57BL/6 donors. (A and B) Percentage of body weight changes and survival after HCT (n = 8). (C) Yield of donor CD4+ T cells from recipient spleen and liver at day 3 after HCT (mean ± SE, n = 4). (D) Three days after HCT, mononuclear cells from the spleen of the BrdU-treated recipients were stained for donor marker H-2k^b, CD4, annexin V, DAPI, or BrdU. Gated donor CD4+ T cells are shown as annexin V versus DAPI or CD4 versus BrdU. One representative is shown of four replicate experiments as well as the mean ± SE of percentage of BrdU^+CD4+ or annexin V^−DAPI^− cells. (E and G) Yield of donor CD4+ T cells from recipient spleen and liver at day 6 after HCT (mean ± SE, n = 4). (F and H) Representative staining patterns and mean ± SE of percentage of BrdU^+CD4+ T cells or annexin V^+CD4+ T cells in the spleen and liver 6 d after HCT (n = 4). *p < 0.05, **p < 0.01, ***p < 0.001.
Lack of host tissue expression of B7H1 results in reduced proliferation of PD-1−/− Tcon cells and amelioration of GVHD. TBI-conditioned BALB/c or B7H1−/− BALB/c recipients were transplanted with CD25−CD8−SPL (0.25 × 10^6) and TCD-BM (2.5 × 10^6) from PD-1−/− C57BL/6 donors.

(A and B) Percentage of body weight changes and survival after HCT (n = 12).

(C and E) Yield of donor CD4+ T cells in the spleen and liver 8 d after HCT.

(D and F) Mononuclear cells from the spleen or liver of the recipients were stained for donor CD4, annexin V, DAPI, or BrdU. The mean (±SE) of percentage of BrdU+CD4+ T cells or annexin V+CD4+ T cells in the spleen or liver are shown (n = 4). Representative flow cytometry staining patterns show gated donor CD4+ T cells in annexin V versus DAPI or CD4 versus BrdU. The mean (±SE) of percentage of BrdU+CD4+ T cells or annexin V+CD4+ T cells in the spleen or liver are also shown (n = 4). *p < 0.05, **p < 0.01.
B7H1/PD-1 interaction intact, as previously described (13, 21). As before, C57BL/6 donor CD25\(^-\)CD8\(^-\)SPL (2.5 \times 10^6) and TCD-BM cells (2.5 \times 10^6) were transplanted into sublethally irradiated BALB/c recipients. Five days after transplantation, recipients were treated daily with anti-B7H1 (43H12) that specifically blocks B7H1/CD80 interaction or rat IgG at a dose of 200 \(\mu\)g/mouse for 2 d. Donor CD4\(^+\) T cell yield, proliferation with BrdU incorporation, and apoptosis in the spleen and liver were measured 24 h after first anti-B7H1 (43H12) injection. (A and B) Percentage of body weight changes and survival after HCT \((n = 10)\). (C and E) Yield of donor CD4\(^+\) T cells in the spleen and liver 6 d after HCT. (D and F) Mononuclear cells from the spleen or liver of the recipients were stained for donor CD4, annexin V, DAPI, or BrdU. The mean (±SE) of percentage of BrdU\(^-\)CD4\(^+\) T cells or annexin V\(^-\)CD4\(^+\) T cells in the spleen or liver are shown \((n = 4)\). (G and H) Donor CD4\(^+\) T cells from the spleen of BALB/c recipients given rat IgG or anti-B7H1 (43H12) Abs were sorted 6 d after HCT, which is 24 h after the first anti-B7H1 injection. Total RNA and protein were isolated and expression level of caspase-3 and Bcl-x\(_L\) were analyzed with real-time PCR (G) and western blot (H). (I) Total RNA was isolated from sorted donor CD4\(^+\) T cells from the spleen of BALB/c recipients given rat IgG or anti-B7H1 (43H12) Abs. Expression level of PD-1 was measured with real-time PCR. The expression levels relative to GAPDH are shown. Means ± SE from three replicate experiments are shown. (J) PD-1 expression on donor CD4\(^+\) T cells was measured with flow cytometry. Gated donor CD4\(^+\) T cells are shown in the histogram of PD-1. The filled gray area represents negative control, and the dashed or solid lines, respectively, represent T cells from recipients treated with anti-B7H1 or control rat IgG. The mean (±SE) of fluorescence of PD-1 is shown; \(n = 4\). *\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\). MFI, mean fluorescence intensity.
after Ab injection. In contrast, ~50% of the control rat IgG–treated recipients survived for >50 d (p < 0.01, Fig. 3A, 3B). The augmentation of GVHD was associated with a marked increase of donor CD4+ Tcon cell proliferation and apoptosis, as judged by BrdU labeling and annexin V staining (p < 0.01, Fig. 3D, 3F). The reduced apoptosis after blockade of B7H1/CD80 interaction was associated with significantly decreased expression of the proapoptotic gene caspase-3, especially with decreased activated/cleaved caspase-3; the reduced apoptosis was also associated with increased expression of the prosurvival gene Bcl-xL, as measured with real-time RT-PCR and Western blot (p < 0.05, Fig. 3G, 3H). Furthermore, specific blockade of B7H1/CD80 interaction resulted in decreased expression of PD-1 as judged by real-time RT-PCR and flow cytometry (p < 0.01, Fig. 3I, 3J). This blocking did not change Tcon cell activation status as judged by their expression of CD69, CD25, CD44, and CD62L (Supplemental Fig. 3B), nor did it impact the expression of CTLA-4 or Fas by donor CD4+ Tcon cells (Supplemental Fig. 3C). These results indicate that B7H1/CD80 interaction augments alloreactive CD4+ Tcon cell proliferation and subsequently promotes apoptosis of the Tcon cells, which is associated with increased expression of PD-1 on the activated T cells.

**Blockade of B7H1/CD80 interaction in the presence of PD-1 reduces IL-2 and increases IL-10 production by activated donor CD4+ Tcon cells**

It was proposed that B7H1-Ig/PD-1 interaction induced T cell IL-10 secretion in an IL-2–dependent manner (3); however, this phenomenon was not observed by another group (4). At that time, the widely accepted ligand for B7H1 was PD-1, and the second ligand CD80 had not yet been identified. To test the impact of B7H1/CD80 interaction on T cell cytokine production, we checked the IL-2 and IL-10 production of the alloreactive CD4+ Tcon cells with or without specific blockade of B7H1/CD80 interaction via injection of anti-B7H1 (43H12). Additionally, we checked T cell production of IFN-γ and TNF-α by intracellular cytokine staining as well as by measuring serum cytokine levels. Interestingly, specific blockade of B7H1/CD80 interaction significantly reduced IL-2 (p < 0.05) but increased IL-10 (p < 0.001) production by alloreactive CD4+ Tcon cells, although no

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**FIGURE 4.** Specific blockade of B7H1/CD80 interaction in the presence of PD-1 reduces IL-2 and increases IL-10 production by activated donor CD4+ Tcon cells. TBI-conditioned BALB/c recipients were given HCT and treated with anti-B7H1 as described in Fig. 3. Twenty-four hours after Ab blockade, spleens were harvested and the percentages of IL-2+, IL-10+, IFN-γ+, and TNF-α+ cells among donor CD4+ Tcon cells were analyzed by intracellular staining. (A) Gated donor CD4+ T cells are shown as CD4 versus IL-2, IL-10, IFN-γ, or TNF-α. One representative of four replicate experiments and the mean (±SE) of percentage of IL-2+, IL-10+, IFN-γ+, and TNF-α+ cells among donor CD4+ T cells are shown. (B) mRNA levels of IL-2, IL-10, IFN-γ, and TNF-α in sorted donor CD4+ T cells 24 h after rat IgG or B7H1 (43H12) treatment were evaluated with real-time PCR. The expression levels relative to GAPDH are shown. Means ± SE from three replicate experiment are shown. (C) Serum levels of IL-2, IL-10, IFN-γ, and TNF-α in rat IgG treatment group and B7H1 (43H12) treatment group were measured with ELISA. The means (±SE) of IL-2, IL-10, IFN-γ, and TNF-α concentration in the serum are shown (n = 4). **p < 0.05, ***p < 0.01, ****p < 0.001.
significant impact on IFN-γ or TNF-α production was observed, as judged by intracellular cytokine staining as well as real-time PCR (Fig. 4A, 4B). Blocking the B7H1/CD80 interaction also significantly reduced serum levels of IL-2 and elevated serum levels of IL-10, IFN-γ, and TNF-α (p < 0.05, Fig. 4C). The increased serum levels of IFN-γ and TNF-α most likely result from an increase of total Tcon cells, because the percentage of IFN-γ– or TNF-α–expressing Tcon cells was not significantly increased after B7H1/CD80 blockade. These results indicate that blockade of B7H1/CD80 interaction reduces IL-2 but augments IL-10 production. It is noteworthy that although blockade of B7H1/CD80 interaction does not impact the percentage of IFN-γ– or TNF-α–producing Tcon cells, serum levels of IFN-γ and TNF-α were elevated and systemic GVHD was augmented owing to the reduced apoptosis and expansion of Tcon cells.

Next, we tested the role of B7H1/CD80 interaction on the proliferation and apoptosis of alloreactive donor CD4+ Tcon cells in the absence of PD-1. Accordingly, BALB/c recipients were transplanted with PD-1−/− donor CD25−CD8−SPL (0.25 × 10⁶) and TCD-BM cells (2.5 × 10⁶). Five days after HCT, the recipients were treated daily with anti-B7H1 (43H12) or control rat IgG (200 μg/mouse) until the entire rat IgG group became moribund. We found that whereas control rat IgG–treated recipients showed rapid body weight loss and all died within 15 d after HCT, anti-B7H1 (43H12)–treated recipients showed slower body weight loss and ∼40% of them survived for >50 d after HCT (p < 0.01, Fig. 5A, 5B).
indicates that blocking B7H1/CD80 interaction in the absence of PD-1 ameliorates acute GVHD. Additionally, we found that 8 d after HCT the donor Tcon cell yield in the spleen and liver of anti-B7H1–treated recipients was significantly reduced (p < 0.05, Fig. 5C, 5E). The proliferation of PD-1−/− Tcon cells in the spleen and liver was also significantly reduced (p < 0.001, Fig. 5D, 5F), but no significant difference in apoptosis was observed in either spleen or liver after anti-B7H1 treatment (Fig. 5D, 5F). These results indicate that B7H1/CD80 interaction in the absence of PD-1 augments Tcon cell proliferation with no effect on T cell apoptosis.

Furthermore, anti-B7H1–treated PD-1−/− alloreactive CD4+ Tcon cells had reduced production of IL-2 and TNF-α (p < 0.01), but no significant changes in their IL-10 or IFN-γ production (Fig. 6A). Anti-B7H1 treatment reduced serum levels of IL-2, IFN-γ, and TNF-α (p < 0.05), although there was no significant impact on serum IL-10 (Fig. 6B). These results indicate that specifically blocking B7H1/CD80 interaction in the absence of PD-1 expression on activated donor T cells can reduce alloreactive Tcon cell proliferation and their production of IL-2 and TNF-α, resulting in reduced serum levels of IL-2 and TNF-α and amelioration of acute GVHD.

Finally, we tested the role of B7H1/CD80 interaction in regulating the Tcon cell proliferation and apoptosis mediated by B7H1/PD-1 interaction in an in vitro MLR assay. Accordingly, sorted CD4+ Tcon cells (0.1 × 10^6) from WT or PD-1−/− donor-type C57BL/6 mice were stimulated with CD11c+ DCs (0.1 × 10^6) from WT or B7H1−/− BALB/c mice. The Tcon cell proliferation and apoptosis, the Tcon cell expression of PD-1, and the concentration of IL-2 in the culture were measured at 3 d after culture.

We found that C57BL/6 WT Tcon cells had significantly increased proliferation and apoptosis in response to stimulation by DCs from WT mice as compared with stimulation by DCs from B7H1−/− BALB/c mice (p < 0.01, Fig. 7A). The increased apoptosis of WT Tcon cells in response to stimulation by WT DCs was associated with higher concentrations of IL-2 in the culture medium and high-level expression of PD-1 on the T cells (p < 0.001, Fig. 7B). Alternatively, the PD-1−/− Tcon cells had significantly stronger proliferation (p < 0.01, Fig. 7C) but no difference in apoptosis in response to WT DCs, as compared with stimulation by B7H1−/− DCs (Fig. 7C). These results indicate that the in vivo observations that B7H1/CD80 interaction augments Tcon cell apoptosis mediated by B7H1/PD-1 and that the augmentation by B7H1/CD80 interaction is associated with increased IL-2 production and increased PD-1 expression by the Tcon cells can be recapitulated by in vitro MLR assays.

**FIGURE 6.** Anti-B7H1–treated PD-1−/− alloreactive CD4+ Tcon cells have reduced production of IL-2 and TNF-α, but no significant changes in their IL-10 or IFN-γ production. TBI-conditioned BALB/c recipients were given HCT and anti-B7H1 treatment as described in Fig. 4. Three days after Ab blockade spleens were harvested and the percentages of IL-2+, IL-10+, IFN-γ+, and TNF-α+ cells among donor CD4+ Tcon cells were analyzed by intracellular staining. (A) Gated donor CD4+ T cells are shown as CD4 versus IL-2, IL-10, IFN-γ, or TNF-α. One representative of four replicated intracellular staining experiments and the mean (±SE) of percentage of IL-2+, IL-10+, IFN-γ+, and TNF-α+ cells among donor CD4+ T cells are shown. (B) Serum levels of IL-2, IL-10, IFN-γ, and TNF-α in rat IgG treatment group and B7H1 (43H12) treatment group were measured with ELISA. The means (±SE) of IL-2, IL-10, IFN-γ, and TNF-α concentration in the serum are shown (n = 4). **p < 0.01, ***p < 0.001.
drodynamic injection, reached peak expression by 24 h, and returned to baseline level by 7 d after injection (Fig. 8A.). Serum B7H1-Ig levels reached $\sim 50$ mg/ml as early as 24 h after injection and plateaued ($\sim 125$ mg/ml) at 7 d after injection. Serum B7H1-Ig persisted for 3 wk and gradually declined to baseline 1 mo following hydrodynamic injection (Fig. 8B).

Five days after hydrodynamic injection of B7H1 or control plasmid, which was 2 d before the peak time point of serum B7H1-Ig levels, recipients were given sublethal TBI and transplanted with CD25$^+$ CD8$^+$ SLP (2.5 or $10^6$) from WT or PD-1$^+$ donor-type C57BL/6 mice. Three days after culture, cells were harvested and stained for CD4, annexin V, and PD-1. Concentrations of IL-2 in the supernatant were also measured. (A) Gated WT CD4+ Tcon cells are shown as CFSE versus annexin V. (B) PD-1 expression level of CD4+ Tcon cells and IL-2 concentration in supernatant. (C) Gated PD-1$^+$ CD4$^+$ Tcon cells are shown as histogram of CFSE or DAPI versus annexin V. One representative flow cytometry pattern is shown of three independent experiments with triplicate wells. Mean $\pm$ SE of nine samples in each group is shown. *p < 0.05, **p < 0.01, ***p < 0.001.

Five days after hydrodynamic injection of B7H1 or control plasmid, which was 2 d before the peak time point of serum B7H1-Ig levels, recipients were given sublethal TBI and transplanted with CD25$^+$ CD8$^-$ SLP (2.5 or $0.25 \times 10^6$) and TCD-BM cells (2.5 or $10^5$) from WT or PD-1$^+$ C57BL/6 donors as described above. First, we compared the recipients given WT donor cells. Whereas recipients treated with control plasmid all developed severe acute GVHD with body weight loss and died within 7 d, the recipients treated with B7H1-Ig plasmid showed a marked reduction in GVHD severity, and $\sim 50\%$ of them survived for $>50$ d with only moderate signs of GVHD ($p < 0.01$, Fig. 8C, 8D). The reduction of GVHD in B7H1 plasmid–treated recipients was associated with marked reduction of donor CD4$^+$ T cell yield in the spleen and liver ($p < 0.001$, Fig. 8E, 8G). The proliferation and apoptosis of donor CD4$^+$ Tcon cells were markedly augmented in the recipients treated with B7H1 plasmid ($p < 0.001$, Fig. 8F, 8H). These results indicate that B7H1-Ig can augment T cell proliferation and apoptosis. This effect may result from agonistic B7H1-Ig interactions with CD80 and PD-1 on activated T cells, as our previous publication showed that the same B7H1-Ig was able to augment T cell proliferation in vitro (33).

In contrast, recipients given PD-1$^+$ donor cells showed opposite results with regard to GVHD. Recipients treated with B7H1-Ig plasmid showed rapid body weight loss and all died within 15 d after HCT, whereas control plasmid–treated recipients showed slower body weight loss and $\sim 40\%$ of them survived for $>50$ d after HCT treatment ($p < 0.01$, Fig. 9A, 9B). The increased severity of acute GVHD in B7H1-Ig plasmid recipients was associated with increased proliferation and yield of PD-1$^+$ donor CD4$^+$ Tcon cells in the spleen and liver ($p < 0.05$, Fig. 9C, 9E).
FIGURE 8. B7H1-Ig secreted by hepatocytes after hydrodynamic injection of B7H1 plasmid ameliorates acute GVHD by augmenting apoptosis of donor CD4+ Tcon cells in the presence of PD-1. B7H1−/− BALB/c mice were given hydrodynamic injection of B7H1 plasmid at a concentration of 10 μg/ml in PBS at the volume of 10% body weight. Hepatocytes and serum of the treated mice were harvested at indicated time points. (A) Relative mRNA expression level of B7H1 plasmid in hepatocytes (n = 4). (B) Serum levels of B7H1-Ig measured with ELISA (n = 4). (C and D) Five days after hydrodynamic injection of B7H1 or control plasmid, the B7H1−/− BALB/c mice were conditioned with TBI and transplanted with CD25−CD8−SPL (2.5 × 10^6) and TCD-BM cells (2.5 × 10^6) from GFP-Foxp3 C57BL/6 donors. Recipients were monitored for body weight changes and survival (n = 8). Six days after HCT, yield of donor CD4+ T cells in the spleen and liver as well as donor CD4+ T cell proliferation with BrdU labeling and apoptosis were measured as described above. (E–H) Six days after HCT, mononuclear cells from the spleen or liver of the recipients were stained for H-2Kb, CD4, annexin V, DAPI, or BrdU. (E and G) Yield of donor CD4+ T cells from spleen and liver of recipients at day 6 after HCT (n = 4). (F and H) Mean (±SE) of percentage of donor BrdU+CD4+ T cells among donor CD4+ T cells in the spleen or liver (n = 4). *p < 0.05, **p < 0.01, ***p < 0.001.
Although no significant difference in donor T cell apoptosis was observed (Fig. 9D, 9F). Taken together, these results further demonstrate that B7H1/CD80 interaction augments proliferation of activated T cells, and this interaction subsequently promotes T cell apoptosis mediated by B7H1/PD-1 interaction.

**Discussion**

We have demonstrated with an acute GVHD model and an in vitro MLR assay of alloimmune response an integrated interaction between the B7H1/CD80 and B7H1/PD-1 pathways. We propose that B7H1/CD80 interaction augments alloreactive CD4+ Tcon cell apoptosis mediated by B7H1/PD-1. As depicted in the summary diagram (Fig. 10), first, after HCT, the alloreactive donor Tcon cells induce GVHD, even though there are interactions between B7H1 on host tissue cells with CD80 and PD-1 on the activated donor Tcon cells (Fig. 10A). Second, lack of B7H1/CD80 interaction in the presence of PD-1 reduces alloreactive Tcon cell proliferation and apoptosis, but the reduction of apoptosis outweighs reduction of proliferation, resulting in expansion of alloreactive Tcon cells and worsening GVHD (Fig. 10B). Third, B7H1/CD80 interaction in the absence of PD-1 augments Tcon cell proliferation with no impact on Tcon cell apoptosis and worsens GVHD (Fig. 10C); in contrast, in the absence of PD-1, blockade of B7H1/CD80 interaction reduces Tcon cell proliferation and reduces Tcon expansion and ameliorates GVHD (Fig. 10D). Consistently, agnostic B7H1-Ig that engages both PD-1 and CD80 on activated T cells augments alloreactive CD4+ Tcon cell proliferation and apoptosis in the presence of PD-1 and ameliorates GVHD; in contrast, B7H1-Ig augments Tcon cell proliferation without affecting T cell apoptosis in the absence of PD-1 and worsens GVHD.

We conclude that B7H1/CD80 interaction augments T cell apoptosis when the B7H1/PD-1 interaction is intact, and this reveals an important interaction between the B7H1/CD80 and B7H1/PD-1 pathways. Previous reports showed that blockade of B7H1/CD80 interaction prevented induction of oral tolerance (21); blockade of this interaction also accelerated diabetes development in NOD mice (20). Alternatively, it has been reported that aged PD-1−/− C57BL/6 mice spontaneously develop characteristic lupus-like proliferative arthritis and glomerulonephritis (44), but no reports of spontaneous autoimmunity in CD80−/− C57BL/6 mice have been made. These observations indicate that B7H1/CD80 interaction augments T cell apoptosis.
CD80 plays an important role in T cell tolerance; however, this interaction is not sufficient in the absence of PD-1 in maintaining T cell tolerance. On the contrary, B7H1/CD80 interaction augments T cell proliferation and augments immunity in the absence of B7H1/PD-1 interaction. Consistently, blockade of B7H1/PD-1 interaction alone is sufficient to break tumor immune tolerance and augment antitumor immunity (24).

Our data suggest that IL-2 may be a link between the B7H1/CD80 and B7H1/PD-1 pathways. We observed that blocking the B7H1/CD80 interaction by use of anti-B7H1 (43H12) that only blocks the B7H1/CD80 interaction reduces alloreactive CD4+ Tcon cell proliferation and production of IL-2 and reduces expression of PD-1 and apoptosis. These observations are consistent with previous reports that IL-2 augments T cell proliferation and induces T cell expression of PD-1, which then mediates T cell apoptosis (45–47). Although Fas/FasL and CTLA-4/B7 interactions augmented activated T cell apoptosis (43, 48), and B7H1-transfected tumor cells induced activated T cell apoptosis partially via Fas/FasL interaction (15), blockade of B7H1/CD80 interaction did not have a significant impact on CD4+ Tcon cell expression of CTLA-4 or Fas. Thus, our studies have revealed a connection between the B7H1/CD80 and B7H1/PD-1 pathways in CD4+ Tcon cells, and we propose that B7H1/CD80 interaction induces T cell proliferation and IL-2 production. IL-2 then induces T cell expression of PD-1 and subsequently augments T cell apoptosis mediated by B7H1/PD-1 interaction.

Our observations that B7H1/CD80 interaction per se augments T cell proliferation and augments immunity and that B7H1/CD80 interaction augments T cell apoptosis mediated by B7H1/PD-1 interaction provide explanations to previous conflicting reports regarding B7H1 and B7H1-Ig in regulating immune responses. One previous report showed that B7H1-Ig augmented human CD4+ T cell expansion and increased IL-2, IFN-γ, and IL-10 production under stimulation with a suboptimal concentration of anti-CD3 in culture (3). In contrast, another report showed that B7H1-Ig reduced human and mouse CD4+ T cell expansion and reduced IFN-γ and IL-10 production by the CD4+ T cells under stimulation with an optimal concentration of anti-CD3 in culture (4). This conflict in reports can be explained by this hypothesis: Upon optimal anti-CD3 stimulation, T cells rapidly upregulate both PD-1 and CD80, and an interaction of B7H1-Ig with PD-1 and CD80 leads to rapid T cell apoptosis and little cytokine production. In contrast, under suboptimal anti-CD3 stimulation, T cells do not upregulate expression of PD-1, and B7H1-Ig interacts predominantly with CD80 on Tcon cells, leading to T cell proliferation and cytokine production with little apoptosis. It was also reported that transgenic expression of B7H1 by islet β cells was found to induce insulitis in nonautoimmune mice (29). This can be explained by this hypothesis: Naïve T cells express CD80 and upregulate its expression after activation (13). Naïve T cells do not express PD-1 but are induced to express PD-1 upon activation (2, 9–12). In nonautoimmune mice, resting autoreactive T cells may express CD80 with little expression of PD-1, and transgenic B7H1 expressed on β cells may interact with CD80 on T cells and augment activation and expansion of the autoreactive T cells, resulting in insulitis.

It seems clear that B7H1 interactions with CD80 and PD-1 have different outcomes in Tcon and Treg cells. Although we observed in the present study that B7H1/CD80 interaction augmented Tcon cell apoptosis in the presence of PD-1, we recently reported that B7H1/CD80 interaction increased survival of Treg cells in the presence of PD-1 (13). Additionally, B7H1/PD-1 interaction was reported to augment conversion of human Th1 cells into Foxp3+ Treg cells (49). We also observed that B7H1-Ig produced by hepatocytes after hydrodynamic injection of B7H1 plasmid augmented Treg cell expansion, although the same B7H1-Ig inhibited Tcon cell expansion (data not shown). Our studies indicate that agonist B7H1-Ig can be a powerful reagent for downregulating autoimmune and alloimmunity of graft rejection and GVHD, that is, when it is administered appropriately.

In conclusion, B7H1/CD80 interaction augments CD4+ T cell apoptosis mediated by B7H1/PD-1 interaction, but in the absence of PD-1, B7H1/CD80 interaction per se augments T cell proliferation and immunity. Agonist B7H1-Ig capable of binding both PD-1 and CD80 can effectively augment T cell activation-induced apoptosis mediated by B7H1/PD-1; however, in the absence of...
References


Supplemental Figures and legends:

Figure S1: Depletion of CD25$^+$ and CD8$^+$ cells in transplants. Foxp3-GFP knockin C57BL/6 mice were used as donors. Spleen cells were stained with anti-CD25 and anti-CD8 mAb, and then CD25$^+$ and CD8$^+$ cells were depleted by MACs columns. A: Comparison of percentage of GFP-FoxP3$^+$, CD8$^+$ in transplants before and after depletion. Samples from each experiment are checked. One representative pattern is shown of more than eight experiments (N>8). B: Comparison of percentage of GFP-Foxp3$^+$ cells among donor CD4$^+$ T cells before and 6 days after transplantation (N>8).
Figure S2: Percentage of survival of recipients given WT or PD-1⁻/⁻ donor CD4⁺ T cells. TBI-conditioned BALB/c recipients were injected with CD8⁻CD25⁻ splenocytes (2.5X10⁶) and TCD-BM (2.5X10⁶) from PD-1⁻/⁻ or WT donors. Percentage of survival after HCT is shown (n=8).
Figure S3: Anti-B7H1 treatment does not down-regulate donor CD4⁺ T cell expression of CTLA-4 or Fas. TBI-conditioned BALB/c recipients were transplanted with CD25⁻CD8⁻ splenocytes (2.5X10⁶) and TCD-BM (2.5X10⁶) from C57BL/6 donors. A: Three and five days after transplantation, the spleen cells were stained for H-2Kᵇ, CD4, CD44, and CD62L. Gated H-2Kᵇ⁺CD4⁺ cells are shown as CD62L versus CD44. One representative is shown of 4 replicate experiments. B: Five days after HCT, recipients were treated with anti-B7H1 (43H12) or Rat IgG. 24 hours after treatment, the recipient spleen cells were harvested and stained for H-2Kᵇ, CD4, CD25, CD69, for H-2Kᵇ, CD4, CD44, and CD62L or for H-2Kᵇ, CD4, CTLA-4, and Fas is shown. One representative pattern of histogram of CD25, CD69 or CD62L versus CD44 of gated CD4⁺H-2Kᵇ⁺ cells (N=4). C: One representative histogram of CTLA-4 and Fas of gated CD4⁺H-2Kᵇ⁺ cells is shown of 3 replicate experiments.